Crystal structures of 70S ribosomes bound to release factors RF1, RF2 and RF3
Jie Zhou, Andrei Korostelev1, Laura Lancaster and Harry F Noller

Termination is a crucial step in translation, most notably because premature termination can lead to toxic truncated polypeptides. Most interesting is the fact that stop codons are read by a completely different mechanism from that of sense codons. In recent years, rapid progress has been made in the structural biology of complexes of bacterial ribosomes bound to translation termination factors, much of which has been discussed in earlier reviews [1–5]. Here, we present a brief overview of the structures of bacterial translation termination complexes. The first part summarizes what has been learned from crystal structures of complexes containing the class I release factors RF1 and RF2. In the second part, we discuss the results and implications of two recent X-ray structures of complexes of ribosomes bound to the translational GTPase RF3. These structures have provided many insights and a number of surprises. While structures alone do not tell us how these complicated molecular assemblies work, is it nevertheless clear that it will not be possible to understand their mechanisms without detailed structural information.

Three key questions
The unexpected finding that recognition of stop codons is mediated by protein factors, rather than tRNAs, raised three fundamental questions concerning the termination mechanism. First, do the release factors themselves directly recognize the stop codons, or do they indirectly mediate recognition, for example through interactions with ribosomal RNA [9–13]? Second, is the hydrolysis reaction catalyzed by the factors themselves, or is the peptidyl transferase activity of the ribosome subverted in the presence of the factor to use water, rather than aminoacyl-tRNA, as an acceptor [14,15]? A third key question is how the very high accuracy of stop codon-dependent termination (which has an error frequency on the order of $10^{-5}$) is maintained in the absence of any proof-reading mechanism. Recent crystal structures of all four possible termination complexes [16–19] have provided clear answers to the first two questions and a solid basis for thinking about the third. The factors and corresponding mRNA stop codons in the four complexes are RF1-UAA (solved at 3.2 Å resolution) [16], RF2-UAG (at 3.0 Å) [17], RF1-UAG (at 3.6 Å) [18] and RF2-UGA (at 3.5 Å) [19].

Structures of the class I release factors RF1 and RF2 bound to the ribosome
The discovery of stop codons, the triplet signals in mRNA that specify translation termination, originated from genetic experiments aimed at characterization of nonsense mutations nearly a half century ago [6,7]. The type I release factors RF1 (specific for UAG and UAA) and RF2 (specific for UGA and UAA) were discovered shortly thereafter [8]. Remarkably, clear answers to the key questions surrounding the mechanism of termination have only begun to emerge in the past few years.

Stop codon recognition
The high-resolution structures clearly show that stop codons are recognized directly by release factors (Figure 2). The conformation of the stop codons in the A site of termination complexes differs strikingly from that of sense codons [22,23]. The first two bases are stacked on each other, but the plane of the third base lies orthogonal to those of the first two in a separate binding pocket (Figure 1a). The putative ‘tripeptide anticodon’ motifs PA/VT (for RF1) and SPF (for RF2)
do indeed participate in codon recognition; however, only a single amino acid (Thr or Ser, respectively) in each motif is directly involved, and they are primarily responsible for discrimination of the 2nd base of the stop codon (Figure 2b,d). Thr186 of RF1, positioned between U1 and A2, donates an H-bond to O4 of U1, and so can only accept an H-bond from adenine, whereas Ser206 of RF2 can both donate and accept H-bonds with the Watson–Crick edges of adenine or guanine. To recognize the third base, RF1 uses a combination of Thr194 and Gln181 to discriminate either A or G via their Hoogsteen edges (Figure 2c). In RF2, there is a Val at the position corresponding to Gln181, restricting the 3rd position to adenine. The first base is universally restricted to U by backbone elements at the N-terminal end of helix α5, where a peptide carbonyl oxygen H-bonds to N3 of the uridine, and a peptide amide NH H-bonds to its O4. This finding explains the failure to obtain mutations that result in altered recognition of the first position of stop codons.

**Catalysis of peptidyl-tRNA hydrolysis**

The universally conserved GGQ motif in domain 3 is positioned next to A76 at the 3’ terminus of P-site tRNA (Figures 1c, 3). In spite of its conservation, mutation of Gln230 in the GGQ sequence to most other amino acids has only a modest effect on catalysis, while mutations of G228 or G229 confer severe decreases in activity [24]. Consistent with this finding, the side-chain of Gln230 is oriented away from the predicted position of the scissile bond. Instead, the backbone NH group of Gln230 is positioned within H-bonding distance of the 3’-hydroxyl of A76. It has been proposed that this backbone NH group plays a dual role in catalysis of the hydrolytic reaction, in stabilization of the tetrahedral transition-state intermediate by H-bonding to the developing oxyanion and in product stabilization by H-bonding to the 3’-OH of the decacylated tRNA (Figure 3b–e) [16]. This mechanism was tested by mutation of glutamine 230 to proline, the sole mutation that is able to eliminate the backbone...
a-NH group [17]. Uniquely among all tested mutations of Gln230, the proline mutation completely abolished catalysis by RF1. The possibility that the effects of the proline substitution were due to a conformational change in the backbone of the GGQ region seems unlikely, since the backbone torsion angles of Gln230 fall well within the range of values commonly observed for proline. Participation of a backbone NH group in stabilization of an oxyanion transition state has precedent in the mechanisms of esterases [25] and proteases [26] as well as in the Ras-catalyzed hydrolysis of GTP [27].

What is the role of the Gln230 side chain? In contrast to the comparatively modest effects on the rate of the hydrolytic reaction, there is a striking effect of mutation of Gln230 on the ability of the factor to discriminate...
against nucleophiles other than water [24]. For the Ser, Ala and Gly mutants, the rate of aminolysis by hydroxylamine was increased by a factor of 15-fold to 170-fold. On the basis of their findings, Shaw and Green proposed that the glutamine side chain excludes nucleophiles bulkier than water, and in addition positions water for in-line attack on the substrate. This proposal is consistent with the available structural information, including the structure of a pre-termination-like complex containing a peptidyl-tRNA analogue bound to the P site [28].

**Coupling of peptidyl-tRNA hydrolysis to stop codon recognition**
Premature hydrolysis of the peptidyl-tRNA ester linkage before recognition of an authentic stop codon is of necessity a rare event. It seems likely that this is accomplished...
by preventing productive docking of the GGQ sequence of domain 3 in the PTC until stop codon recognition is established. In the crystal structures of isolated RF1 and RF2 [29,30], domains 2 and 3 are folded into a compact state that differs dramatically from that of the extended conformations seen in their respective termination complexes [16–19,31–33]. The possibility of interconversion of the compact and extended forms is supported by direct observation of both closed and open conformations of free release factors in solution [34,35]. Comparison of the ribosome-bound structures with those of the free factors suggests that a key event involves rearrangement of a loop (residues 286–300) connecting domains 3 and 4 that we term the ‘switch loop’ (Figure 1d), which extends helix α7 by two helical turns and directs domain 3 into the PTC (Figure 1c). The rearranged state of the switch loop appears to be stabilized by extensive hydrogen bonding and packing interactions within a pocket formed by protein S12, the 530 loop of 16S rRNA, the β-sheet of domain 2 and the flipped nucleotides A1492 and A1913, which form the floor of the pocket [16,18]. The extra turns of helix formed at the end of α7 are held in position by contacts with the β-sheet of domain 2 of RF1 and the shifted loop of helix 69 of 23S rRNA.

Rearrangement of the switch loop and fixing the orientation of the extended α7 helix depend on interactions that are only possible following stop codon recognition, which include conformational changes involving A1492 and A1493 of 16S rRNA and A1913 of 23S rRNA. Flipping out of A1493 or failure of A1913 to pack on A1493 in h44 (as seen for sense codon recognition) would not only result in steric clash between these nucleotides and domain 2 of RF1, preventing correct docking of its reading head on the stop codon, but would also prevent formation of the binding pocket for the rearranged switch loop and other contacts needed to position the extended α7 helix, thus docking the GGQ into the PTC. This proposal would account for the stringent dependence of peptidyl-tRNA hydrolysis on stop codon recognition. The crucial role played by A1913 would also explain the deleterious effects on translation termination caused by deletion of helix 69 of 23S rRNA [36].

Structures of the class II release factor RF3 bound to the ribosome
The class II release factor RF3 was initially identified by its stimulatory effects on the activity of class I release factors [37]. It is now believed that its role is in dissociating the class I release factors from the ribosome following termination [38,39]. RF3 is of special interest because it is a GTPase structurally related to EF-G, and like EF-G, promotes intersubunit rotation of the ribosome [40,41]. Thus, the crystal structures of its complexes with the ribosome will not only help to understand its own mechanism of action, but may also provide insight into how interactions with the translational GTPases affect the structural dynamics of the ribosome.

RF3-dependent structural changes in the ribosome
Cryo-EM reconstructions showed that RF3 binds to the ribosome in the same location as observed for EF-G and EF-Tu, and induces intersubunit rotation similar to that of EF-G [40,42]. Recently, two crystal structures of RF3-ribosome complexes have been reported, one of *Thermus thermophilus* ribosomes complexed with *Escherichia coli* RF3-GDPCP [28] and the other of *E. coli* ribosomes with *E. coli* RF3-GDPNP [43]. Along with the structure of a complex containing the ribosome recycling factor RRF [44], these represent the first crystal structures showing the 70S ribosome trapped in a rotated state. The RRF and *T. thermophilus* RF3 structures show a similar intersubunit rotation, relative to the classical state, of about 9°, a 30S head rotation of 3–4° and contain a deacylated tRNA bound in the P/E hybrid state. In contrast, the *E. coli* RF3 structure has an intersubunit rotation of about 7° and a striking 14° rotation of the 30S head (Figure 4a,b).

Both of the major rotational events result in alteration or disruption of intersubunit bridges. In general, the RNA-rich bridges nearer to the center of rotation at bridge B3 are preserved in the rotated state, made possible by bending, rotation or compression of local elements of rRNA. The peripheral bridges, such as bridge B1a, where displacements of more than 30 Å can occur, are disrupted and replaced by new contacts.

Structural changes in RF3
RF3 binds to the ribosome at the entry to the intersubunit cavity (Figure 4c) in a position that overlaps with that of EF-G and EF-Tu. Domain I contacts the 30S subunit at the sarcin–ricin loop (SRL) of 23S rRNA and protein L6. Domains II and III contact the 30S subunit at helices h5 and h15 of 16S rRNA and protein S12. Compared with the crystal structure of free RF3 solved in its GDP form [42], the ribosome-bound structures of RF3-GDPCP and RF3-GDPNP are dramatically rearranged (Figure 4d,e). Domain II undergoes a 7° rotation and domain III rotates by 55° [43].

In the GTP-binding domain I, switch loop I, which is conserved among G proteins and disordered in all previous structures of RF3 and EF-G, becomes ordered, forming a cage around the bound GTP analogue (Figure 5a,b). The histidine residue previously proposed to activate the attacking water molecule in catalysis of the GTPase reactions of all ribosomal G proteins, based on the structure of the EF-Tu-GDPCP-ribosome complex [45], is located more than 8 Å from the γ-phosphate, placing it out of range for direct participation in catalysis (Figure 5c). Electron density for a presumed magnesium ion is found next to the β-γ oxygen, which could play a catalytic role by stabilizing the oxyanion in the transition state (Figure 5d).
Implications for the functions of RF3 and EF-G

Following peptidyl-tRNA hydrolysis, RF1/2 remain tightly bound to the ribosome. The observed requirement for deacylation of peptidyl-tRNA for RF3 to effect dissociation of RF1 and RF2 [46] can be explained by allowing P/E hybrid-state formation and therefore inter-subunit rotation. The RF3-induced rotated state creates clash between domain IV of RF1/2 and the head of the...
30S subunit, and between domain I of RF1/2 and the L11 stalk of 23S RNA. Thus, both large-scale rotational movements of the ribosome would contribute to destabilization of binding of release factors RF1 or RF2. But these same clashes would interfere with binding of RF3 to a post-termination complex in its GTP state. One possibility is that RF3 binds initially in its GDP form followed by exchange of GTP for GDP [46]. However, RF3 GDP is not observed to bind stably to the termination complex [28, 43, 47]. An alternative possibility is that the termination complex samples the rotated state, which is stabilized by binding of RF3-GTP, causing dissociation of RF1 or RF2.

One unexplained puzzle in the binding of EF-G to the ribosome is that domain IV of EF-G occupies the same space as A-site tRNA, yet these two ligands must coexist on the ribosome during initial binding of EF-G. In the E. coli structure of the RF3-ribosome complex, domain I of RF3 is rotated by ~45° relative to that of EF-G-GDP or EF-Tu. Docking of EF-G in the RF3 orientation results in positioning of its domain IV near the shoulder of the 30S subunit, relieving potential clash with A-site tRNA [43] (Figure 6a,b). This orientation resembles an earlier cryo-EM reconstruction of an EF-G-ribosome complex trapped in the pre-translocation state in the presence of thiostrepton [48]. Further studies on trapped intermediates containing EF-G will be needed to resolve this confusing aspect of the translocation mechanism.

Another problem that must be addressed in thinking about the translocation mechanism was recognized by Cate and co-workers [49]. In moving from the 30S P site
to the E site, the anticodon stem-loop (ASL) must pass through a channel between the head and platform that is constricted to a width of only $13-14\,\text{Å}$ in the classical state of the 70S ribosome. Clearly, one or both of these features must move to enable translocation of P-site tRNA, and the latter authors noted that this could be accomplished by rotation of the head of the small subunit as seen in a cryo-EM reconstruction of an 80S ribosome complex [50]. Sufficiently large rotational movements of the head have been observed in cryo-EM [51,52] and crystal [53] structures. Spahn and coworkers [51] have proposed the existence of additional hybrid intermediate states involving head and body rotation in EF-G-catalyzed translocation. In the $E.\ coli$ RF3-ribosome complex [43], simultaneous large-scale rotations of both the 30S body (7°) and head (14°) are observed. The head rotation opens the constricted channel from 14 to 22 Å, sufficient to allow translocation of the ASL. Moreover, the net displacement of the anticodon stem contacts G1338 and A1339 in the 30S subunit head, due to combined body and head rotations, is about 23 Å, corresponding almost exactly to the distance traveled by the ASL during movement from the P to the E site (Figure 6c,d). These findings may have implications for the mechanism of EF-G-dependent translocation.

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